

REMARKS

Claims 45-114 remain in the application. Claims 88-111 have been withdrawn as a result of an earlier restriction requirement. Claims 48, 63, 64, 74, 75, 77-79, 83, 87 and 112 have been amended for clarity without any prejudice or disclaimer of any previously claimed subject matter.

IDS

Applicants re-submit, along with this response, a copy of the supplemental IDS and the Menon *et al.* reference, which was filed on September 26, 2001. The submitted IDS contains the correct date for the Menon *et al.* reference and was not considered by the Examiner. This reference and its correct date (1997) were previously submitted on September 26, 2001 and no fee is believe to be due. Applicants respectfully requests that the Examiner fully reconsider this reference.

Objection to Claims

Claim 112 was objected to for claiming a non-elected subject matter. Applicants have amended claim 112 to address the objected issue. Thus Applicants respectfully request withdrawal of this objection.

Rejection Under 35 U.S.C. §112, First Paragraph

A. Claims 45-51, 53-87 and 112-114

Claims 45-51, 53-87 and 112-114 were rejected under 35 U.S.C. §112, first paragraph as failing to comply with the written description requirement. Applicants respectfully traverse this rejection as follows.

The claimed invention is directed to a fusion protein comprising a fusion partner, in this case rubredoxin, fused directly or indirectly to a protein or peptide of interest, together with methods and materials for producing the fusion protein in a host cell and purifying the fusion protein. Applicants are not required to identify each and every species which encodes for rubredoxin. It is well known in the art that "rubredoxins from

several different anaerobic organisms have been discovered and characterized.”
(Specification, page 6, lines 28-29). Applicants have developed a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein. The exact origin of the rubredoxin is not critical to the nature of the invention. Rubredoxin encoded from any well known source such as *Desulfovibrio vulgaris*, *Desulfovibrio gigas*, *Desulfovibrio desulfuricans*, strain 27774, *Clostridium pasteurianum*, *Archaeon Pyrococcus furiosus*, *Guillardia theta* or *Pyrococcus furiosus* may be used in accordance with the claimed invention. (See also Specification on page 9, lines 25-31: “*Desulfovibrio*, *Clostridium*, *Deulfoarculus* or *Pyrococcus* spp., more preferably from *D. vulgaris*, *D. vulgarius* (Hildenborough), *C. pasteurianum*, *C. butyricum*, *D.baarsii* or *P.furiosa*...D76419 (rub gene for *D. vulgaris*), M28848 (rub gene for *D. vulgarius* (Hildenborough), M60116 (*C. pasteurium* rubredoxin gene), Y11875 (*C. butyricum* rubredoxin gene), and X99543 for *D. baarsii*.”). Applicants have also identified in the table below a handful of prior art which discloses rubredoxin from various sources which were known at the time of the invention. We have enclosed copies of these reference for the Examiner’s convenience.

Crystallographic study of rubredoxin from the bacterium <i>Desulfovibrio desulfuricans</i> strain 27774. <u>Sieker LC, Jensen LH, Prickril BC, LeGall J</u> <i>J Mol Biol</i> 1983 Nov 25 171:1 101-3
Purification and properties of ferredoxin and rubredoxin from <i>Butyribacterium methylotrophicum</i>. <u>Saeki K, Jain MK, Shen GJ, Prince RC, Zeikus JG</u> <i>J Bacteriol</i> 1989 Sep 171:9 4736-41
Expression of a synthetic gene coding for the amino acid sequence of <i>Clostridium pasteurianum</i> rubredoxin. <u>Eidsness MK, O Dell SE, Kurtz DM, Robson RL, Scott RA</u> <i>Protein Eng</i> 1992 Jun 5:4 367-71
Synthesis and characterization of <i>Desulfovibrio gigas</i> rubredoxin and rubredoxin fragments. <u>Christensen HE, Hammerstad Pedersen JM, Holm A, Iversen G, Jensen MH, Ulstrup J</u> <i>Eur J Biochem</i> 1994 Aug 15 224:1 97-101
Isolation, characterization, and primary structure of rubredoxin from the photosynthetic bacterium, <i>Heliobacillus mobilis</i>. <u>Lee WY, Brune DC, LoBrutto R, Blankenship RE</u> <i>Arch Biochem Biophys</i> 1995 Apr 1 318:1 80-8
Recombinant two-iron rubredoxin of <i>Pseudomonas oleovorans</i>: overexpression, purification and characterization by optical, CD and ¹¹³Cd NMR spectroscopies.

<u>Lee HJ, Lian LY, Scrutton NS</u> <u>Biochem J 1997 Nov 15 328 (Pt 1): 131-6</u>
--

Although Applicants have illustrated numerous examples for sources of rubredoxin, Applicants are not required to exemplify each and every single suitable source of rubredoxin known. All that is required is that the disclosure of the application as originally filed reasonably conveys to the artisan that the inventor had possession at that time of the presently claimed subject matter. *See In re Kaslow*, 707 F.2d 1366, 1375, 217 U.S.P.Q. 1089, 1096 (Fed. Cir. 1983).

Applicants also direct the Examiner's attention to the enclosed article by Kohli, B.M. and Ostermeier, C., entitled, "A Rubredoxin Based System For Screening Of Proteins Expression Conditions And On-Line Monitoring Of The Purification Process" (Protein Expression and Purification, 28 (2003) 362-367), which discloses spectroscopic properties and the yield of rubredoxin fused to a typical target protein. This research corroborates and supports Applicants' claimed invention, using rubredoxin as colored fusion tag for expression of recombinant proteins. In one experiment," superglowGFP (Qbiogene), *Flv* from *H. pylori*, and Rubredoxin (Rub) from *T. maritime* were individually fused to the C-terminus of the human CDIIc I-domain, expressed in *E. coli*, and purified via N-terminal His-Tag. (Kohli et al, page 364, first paragraph). This research suggests that rubredoxin, as claimed in the present invention, demonstrates "its robustness, practical use in protein quantitation, and tracking as well as its high potential for automated purification." (Kohli et al, page 366, conclusion section.).

Further, the Office Action specifically states that "[t]he specification fails to describe any other representative species by any identifying characteristics or properties other than the functionality of encoding rubredoxin and fails to provide any structure: function correlation present in all members of the claim genus." (Paper No. 16, page 4-5). If the specification is read in light of the knowledge and level of skill in the art, the specification discloses the steps of the claimed process and the elements of the claimed composition. As stated by the Federal Circuit "an inventor is not required to describe every detail of his invention. An applicant's disclosure obligation varies according to the art to which the invention pertains." *In re Hayes Microcomputer Products Inc. Patent*

Litigation, 982 F.2d 1527, 1534-35 (Fed. Cir. 1992). The prior art provides numerous organisms which encode for rubredoxin from a wide variety of bacteria. At the time of the claimed invention, Applicants have stated that “rubredoxin from numerous different organisms have been isolated, and the amino acid sequences of various rubredoxins and the genes encoding various rubredoxins have been published.” (Specification page 25, lines 27-29). In the present application, Applicants disclose the use of *D. vulgaris* as the source for the gene that encodes rubredoxin as a representative example, which are conventional in the art, and are known to one of ordinary skill in the art. The selection of the source for encoding rubredoxin is sufficiently developed so as to put one of skill in the art in possession of the steps of the method and the elements of the composition. In other words, one skilled in the relevant art would understand what is intended by the claimed invention and how to carry it out. In light of the evidence submitted, one of ordinary skill in the art would understand the invention to be related to fusion protein comprising a fusion partner, fused directly or indirectly to a protein or peptide of interest, together with methods and materials for producing the fusion protein in a host cell and purifying the fusion protein, and not critically dependent upon the origin of the rubredoxin in each case. The courts have pointed out that “[n]ot every last detail [of an invention need] be described [in a patent specification], else patent specifications would turn into production specifications, where there were never intended to be.” *In re Gay*, 309 F.2d 769, 774, 135 U.S.P.Q. 311; 316 (C.C.P.A. 1962).

Further, the claimed invention is not directed to a particular polynucleotide sequence; rather, the claims are directed to a construct describing a particular way of combining types of sequences (i.e. a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide of interest). The claims are therefore directed to a novel combination of known types of sequences, and are not drawn to a particular sequence. As such, Applicants are not required to disclose every possible sequence that can be used interchangeably in the claimed polynucleotide constructs. The novelty of the invention does not lie in the particular sequence used, but lies in the structure of the construct. As least for the above mentioned remarks, Applicants respectfully request withdrawal of this rejection.

B. Claims 64 and 82-87

The Examiner has also rejected claims 64 and 82-87 under 35 U.S.C. §112, first paragraph. In particular, the Examiner asserts that specification fails to describe any other representative species of β -amyloid and thus “fails to sufficiently describe the claimed invention in such full, clear, concise, and exact terms that a skilled artisan would recognize that applicants were in possession of the claimed invention.” (Office Action page 5). Applicants respectfully traverse this rejection.

Applicants’ invention in these claims is directed to a method for making a rubredoxin- β -amyloid fusion protein comprising introducing into a host cell a recombinant polynucleotide comprising a nucleotide sequenced encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused β -amyloid peptide, wherein the host cell contains or is supplied with at least one isotopically labeled amino acid or precursor compound; and expressing a rubredoxin- β -amyloid fusion protein in the host cell wherein the fused β -amyloid peptide is uniformly isotopically labeled. Applicants have provided exemplary support of well known β -amyloid such as β -amyloid 1-40 and 1-42 peptides (see Example II, page 29).

Applicants also bring to the Examiner’s attention, in the table below, that at the time of the invention, various forms of β -amyloid have been characterized and are widely known, copies of which are enclosed.

<i>A Beta40 Is A Major Form Of Beta-Amyloid In Nonhuman Primates.</i>
--

Gearing M, Tigges J, Mori H, Mirra SS Neurobiol Aging 1996 Nov-Dec 17:903-8
--

<i>Amyloid Beta Protein In Rat Soleus Muscle In Chloroquine-Induced</i>
--

<i>Myopathy Using End-Specific Antibodies For A Beta 40 And A Beta 42: Immunohistochemical Evidence For Amyloid Beta Protein.</i> Tsuzuki K, Fukatsu R, Takamaru Y, Yoshida T, Hayashi Y, Yamaguchi H, Fujii N, Takahata N Neurosci Lett 1995 Dec 202:77-80
<i>Homology Of The Amyloid Beta Protein Precursor In Monkey And Human Supports A Primate Model For Beta Amyloidosis In Alzheimer's Disease.</i> Podlisny MB, Tolan DR, Selkoe DJ Am J Pathol 1991 Jun 138:1423-35
<i>A Comprehensive Study Of The Spatiotemporal Pattern Of Beta-Amyloid Precursor Protein Mrna And Protein In The Rat Brain: Lack Of Modulation By Exogenously Applied Nerve Growth Factor.</i> Neve RL, Valletta JS, Li Y, Ventosa-Michelman M, Holtzman DM, Mobley WC Brain Res Mol Brain Res 1996 Jul 39:185-97
<i>Permeability And Residual Plasma Volume Of Human, Dutch Variant, And Rat Amyloid Beta-Protein 1-40 At The Blood-Brain Barrier.</i> Poduslo JF, Curran GL, Haggard JJ, Biere AL, Selkoe DJ Neurobiol Dis 1997 4:27-34
<i>Zinc-Induced Aggregation Of Human And Rat Beta-Amyloid Peptides In Vitro.</i> Esler WP, Stimson ER, Jennings JM, Ghilardi JR, Mantyh PW, Maggio JE J Neurochem 1996 Feb 66:723-32

Applicants reiterate that the test for determining compliance with the written description requirement of 35 U.S.C. §112, first paragraph, is whether the disclosure of the application as originally filed reasonably conveys to the artisan that the inventor had possession at that time for the presently claimed subject matter. *See In re Kaslow*, 707 F.2d 1366, 1375, 217 U.S.P.Q. 1089, 1096 (Fed. Cir. 1983). Determining whether that written description requirement is satisfied requires reading the disclosure in light of the knowledge possessed by those skilled in the art. That knowledge can be established by reference to patents and publications available in the public prior to the filing date of the application. *See In re Alton*, 76 F.3d 1168, 37 U.S.P.Q.2d 1578 (Fed. Cir. 1996). In the present case, Applicants have not only sufficiently provided representative β -amyloid, but have also indicated that numerous β -amyloid are widely-known by a skilled artisan at the time of the invention. As argued above, Applicants are not required to list each and every single working example of β -amyloid. Thus, at least for the mentioned arguments, Applicants respectfully request withdrawal of this rejection.

C. *Claim 52*

The Examiner has additionally rejected claim 52 under 35 U.S.C. §112, first paragraph, for non-enablement of pRUBEX3. Applicants respectfully traverse this rejection.

Turning to the rejection itself, the Examiner must first make a *prima facie* case of non-enablement that is well-grounded scientific reasoning or evidence. Applicants remind the Examiner that when rejecting a claim under 35 U.S.C. §112, the Examiner bears “the initial burden or setting forth a reasonable explanation as to why [he/she] believes that the scope of protection provided by [the] claim is not adequately enabled by the description of the invention provided in the specification.” *In re Wright*, 999 F.2d 1557, 1562, 27 U.S.P.Q.2d 1510, 1513 (Fed. Cir. 1993). To object to a specification on the grounds that the disclosure is not enabling with respect to the scope of a claim sought to be patented, the Examiner must provide evidence or technical reasoning substantiating those doubt. *Id.*; *M.P.E.P* §2164.04; and *35 U.S.C. §112 First Paragraph Enablement Training Manuel*. In the present case, the Examiner has only circularly reasoned, without any evidence or reason to doubt the truth of the statements made in the patent application, that claim 52 is non-enabled because pRUBEX3 is not fully disclosed. See *In re Wright*, 999 F.2d 1557, 1562, 27 U.S.P.Q.2d 1510, 1513 (Fed. Cir. 1993) (without a reason to doubt the truth of the statements made in the patent application, the application must be considered enabling). Thus, the Examiner has failed to establish the *prima facie* case of rejection under 35 U.S.C. §112.

Even if the Examiner had established a *prima facie* case for rejecting claim 52 under 35 U.S.C. §112, claim 52, directed to the expression vector which is pRUBEX3, wherein pRUBEX3 comprises a nucleotide sequence encoding an affinity tag having at least one amino acid sequence selected from the group consisting of His-His-His-His-His-His (SEQ ID NO:4) and His-Gly-Leu-His (SEQ ID NO:7), is enabled.

The specification clearly defines pRUBEX3, which comprises a histidine tag and a Factor Xa cleavage site of pRUBEX2 as well as containing “as part of the intervening spacer a portion of the multiple cloning region to facilitate cloning of the nucleotide sequence encoding the fused polypeptide into the vector”. (Specification, page 20, lines

3-7). Applicants have also provided, in Figure 1, a schematic of the vector pRUBEX3, including the Multiple Cloning Region (MCR) and the nucleotide sequence of a portion of pRUBEX3 together with the amino acid sequence encoded. Not only is pRUBEX3 defined and exemplified in Figure 1, Applicants have also provided in Example 1, the method to yield pRUBEX3 (Specification, Example 1, page 25, line 23 to page 28, line 24; **with emphasis** on page 28, lines 23-24). Thus, the specification has been disclosed in a manner that one skilled in the art would be able to practice the invention without an undue amount of experimentation (emphasis added). *See In re Colianni*, 561 F.2d at 224, 195 U.S.P.Q. at 153 ;*see also M.P.E.P* §2164.02.

The Examiner has also relied on 37 C.F.R. §1.802, as a basis for this rejection, which states:

(a) Where an invention is, or relies on, a biological material, the disclosure **may include** reference to a deposit of such biological material.

(b) Biological material need not be deposited unless access to such material is necessary for the satisfaction of the statutory requirements for patentability under **35 U.S.C. 112. Biological material need not be deposited, inter alia, if it is known and readily available to the public or can be made or isolated without undue experimentation.**

(irrelevant sections omitted).

As stated above, Applicants are not required to submit the biological material if it can be made or isolated **without undue experimentation**. Applicants have articulated above that pRUBEX3 have been defined in the specification and exemplified in Figure 1 and Example 1. Because pRUBEX3 would not require an undue amount of experimentation to practice to those skilled in the art, Applicants are not required to deposit this biological material as it has been satisfied under the statutory requirements of 37 C.F.R. §1.802 and 35 U.S.C. §112. *See also Amgen, Inc. v. Chugai Pharm. Co.*, 927 F.2d at 1210, 18 U.S.P.Q.2d at 1024 (inventor did not need to deposit cells used in the invention's best mode if one skilled in the art could reproduce without undue experimentation); *see also Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d at 1385, 231 U.S.P.Q. at 94 (The mere fact that the best mode requires sophisticated competent people to engage in a labor-intensive and time-consuming process does not

amount to a best mode violation in the absence of a deposit. The key to the analysis is whether the scientists must engage in *undue experimentation*).

Applicants have clearly enabled pRUBEX3 and that the Examiner has failed to provide reasonable evidence to establish a *prima facie* case for this rejection, thus, Applicants respectfully request withdrawal of this rejection.

Rejection Under 35 U.S.C. §112, Second Paragraph

A. Claim 48

The Examiner has rejected claim 48 under 35 U.S.C. §112, second paragraph. Applicants have amended claim 48 for clarity and thus respectfully request withdrawal of this rejection.

B. Claim 64

The Examiner has rejected claim 64 under 35 U.S.C. §112, second paragraph. In particular, the Examiner objects to the phrase “biologically active fragment, modification or analogue thereof” and asserts that “analogue” is “neither art defined or specification defined. Applicants have amended claims 64 and 75 for clarity and thus respectfully request removal of the rejection. One skilled in the art would know that a wide variety of biologically active amino acid sequences are encompassed by the term “amyloid peptide” as demonstrated by the great body of knowledge on this protein as described above.

Applicants have also given an illustrative example of such biologically active amyloid peptides such as those found in Alzheimer’s plaque. “For example, tyrosine at the 10 position in β -amyloid (Try10) can be changed to tryptophan to yield a bioactive β -amyloid peptide analogue, and the tryptophan can be detectably labeled using IGTC to generate modified bioactive peptide having a chartreuse color.” (Specification, page 13, lines 13-17). In addition to Try10, Applicants have also shown biological active β -amyloid with arginine at the 5 position.

C. Claims 77, 78, and 79

The Examiner has rejected claims 77, 78, and 79 under 35 U.S.C. §112, second paragraph. Applicants have amended claims 77, 78, and 79 for clarity and thus should no longer be an issue. Thus, Applicants request withdrawal of this rejection.

Rejection Under 35. U.S.C. §102

The Examiner has rejected claims 45-51, 53-60, 64-71 and 76-81 under 35 U.S.C. §102. Applicants respectfully traverse this rejection.

The Examiner asserts that Menon *et al.* teach a DNA encoding a 13.6kD fusion protein comprising a leader protein, a histidine tag sequence, a Factor Xa cleavage site and the 1-40 β -amyloid peptide or 1-42 β -amyloid peptide. The present claimed invention is directed to a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal polypeptide, when not fused to the rubredoxin constituent, is insoluble or forms inclusion bodies in a recombinant expression system. On the other hand, Menon *et al.* does not disclose the use of rubredoxin. The Menon *et al.* reference is merely a published abstract which discloses only the amyloid and is silent to the identity of the fusion partner leader protein in order to avoid disclosing the invention. The Examiner suggests that the rubredoxin has the same molecular weight and iron binding ability as was inherently disclosed. Applicants first point out that the reference does not teach the critical element of one aspect of the claimed invention, i.e. rubredoxin, and thus failed to establish a *prima facie* case of anticipation. Secondly, the Examiner's reliance on molecular weight and iron binding ability to deduce the rubredoxin fusion is misplaced. The size of the fusion construct is 13.6 kD (pg. 31 of patent application), of which rubredoxin is 5.6 kD (pg. 6 of patent application), β -amyloid is ~4.5 kD (different forms of beta-amyloid used), polylinker is 3.5 kD. Thus the size of the undisclosed rubredoxin+polylinker = 9.1 kD. Other proteins that are in this size range, that bind iron, are:

(1) Ferredoxins (6 to 12 kD)
<http://www.chem.uh.edu/Faculty/Pettitt/ResearchWebpages/beck/ferredoxins.html>. Ferredoxins have

been studied for over 30 years, Otaka, E. and Ooi, T (1987) Examination of protein sequence homologies. IV. Twenty seven bacterial ferredoxins. J. Mol. Evol. 26: 257 .

(2) Desulforedoxin (7.9kD),
http://www.chem.uwa.edu.au/enrolled_students/BI_C_sect2/sect2.3.2.2.html.

Therefore, the invention would not have been inherently disclosed in the cited reference, because one of skill in the art would not have conclusively known that the unnamed fusion partner was rubredoxin. Thus, because the cited reference does not teach each and every single aspect of the presently claimed invention, Applicants respectfully request withdrawal of this rejection.

Rejection Under 35 U.S.C. §103

The Examiner has rejected claims 61-63, 72-75, 82-87 and 112-114 under 35 U.S.C. §103(a) as unpatentable over Menon et al., Ueno et al ., and Dobeli et al. Applicants respectfully traverse this rejection.

As discussed above, the presently claimed invention is directed to a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal polypeptide, when not fused to the rubredoxin constituent, is insoluble or forms inclusion bodies in a recombinant expression system. Because the Menon et. al. article fails to identify the leader protein, rubredoxin, and because there are other possible substitutes that are approximately the same size and bind iron, the reference can not support a prior art rejection for inherency or obviousness.

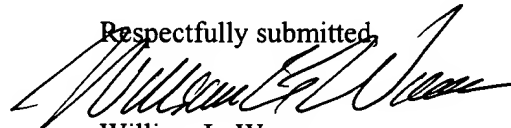
The Dobeli et al paper, where they made a 13.8 kD fusion with the amyloid, is not soluble and goes into the inclusion bodies. Further, nowhere in the reference does it teach or suggest an iron containing nucleotide having red color for easy tracking. Not only is there no suggestion or motivation, the Dobeli et al. reference teaches away from the presently claimed invention. The Dobeli reference teaches a chemical cleavage of beta-amyloid from fusion which causes side reactions. The Ueno et al. reference does not

teach or suggest the use of any β -amyloid. Further the Ueno disclosure does not teach rubredoxin constituent that is soluble, nor does it teach a rubredoxin constituent that goes into the inclusion bodies. Thus, even if all the references were to be combined, there would be no teaching or suggestion of the claimed invention. Prior to the present application, no one had described that rubredoxin was a suitable fusion partner for creating soluble expression products that are also colorimetric. Further evidence of the surprising utility of rubredoxin in this context is that the previously mentioned article published in Kohli et. al., where skilled researcher at Novartis Pharma were surprised to "discover" the subject expression system, not realizing that the invention had already been made at least five years earlier by the present applicants. Thus, Applicants respectfully request withdrawal of this rejection.

In view of the present amendment and response to Office Action, Applicants respectfully request that a timely Notice of Allowance be issued in this case.

Applicants enclose a Petition for a Two Month Extension of Time to reply, to and including, May 16, 2004, and a check in the amount of \$210.00 for the associated fee. No other fee is believed to be required for this Response to Office Action. Although Applicants believe the amount of the fee is correct, the Commissioner is authorized to charge any deficiency or credit any overpayment to Deposit Account 19-5029.

Respectfully submitted,



William L. Warren
Reg. No. 36,714

SUTHERLAND ASBILL & BRENNAN LLP
999 Peachtree Street, NE
Atlanta, Georgia 30309-3996
(404) 853-8000 (main)

Got from Jan Christen.



ACADEMIC
PRESS

Available online at www.sciencedirect.com

SCIENCE @ DIRECT®

Protein Expression and Purification 28 (2003) 362–367

Protein
Expression
& Purification

www.elsevier.com/locate/yprep

A Rubredoxin based system for screening of protein expression conditions and on-line monitoring of the purification process

Bernhard M. Kohli^a and Christian Ostermeier^{b,*}

^a Biocentrum, Basel, Switzerland

^b Novartis Pharma, Lichtstrasse 35, CH-4002 Basel, Switzerland

Received 30 October 2002, and in revised form 29 November 2002

Abstract

Rubredoxin (Rub) from *Thermotoga maritima*, a 6.1-kDa red protein containing an Fe(III)-cysteine₄ center, was evaluated for its usefulness as a colored fusion tag for expression of recombinant proteins in *E. coli*. Here, we describe the Rub features relevant to accelerating screening for optimal high yield soluble expression conditions and automating the ensuing purification process. Spectroscopic properties and the yield of Rub fused to a typical target protein were compared to analogous GFP and Flavodoxin constructs, showing Rub absorption to be sufficient for structural genomics purposes while being produced at much higher soluble levels than GFP constructs. Based entirely on Rub absorption at 380 nm, both generic and affinity purification of crude cell lysate were performed: thus guided anion exchange purification of a Rub fusion construct as well as automated Ni-NTA purification resulted in pure protein. Rub is stable over a wide range of pH, temperature, and buffer environments, enabling robust purification protocols. Across a variety of fusion constructs, including N- and C-terminal Rub, quantitation via the Rub signal was shown to reliably correlate with analytical HPLC data obtained at 220 nm. We propose the “RubyTag” as an alternative to conventional protein fusion tags, as it combines a specific absorption signal with convenient biochemical and biological properties. Further, it allows direct on-line readout on conventional chromatography systems, holding promise for automated multi-step chromatography. © 2002 Elsevier Science (USA). All rights reserved.

Functional and structural genomics and proteomics initiatives generally require mg amounts of homogeneous, soluble, and native protein. While *Escherichia coli* is the workhorse for protein production, preparation of eukaryotic proteins can often be a daunting task. To avoid labor intensive and costly use of eukaryotic expression systems, multiple fusion constructs, expression, and purification conditions can be systematically screened and optimized in *E. coli* [1]. Its rapid growth typically allows production and initial capturing of protein in less than a day. Thus, the analysis of chromatography fractions from a multitude of samples expressed in parallel swiftly turns into a bottleneck.

Rubredoxin is a small, highly soluble bacterial protein [2]. It is extremely stable and well expressed in

E. coli. Fused to the N- or C-terminus of a target protein, it confers a unique red color that is directly detectable during typical protein handling processes. We describe here the use of a Rubredoxin color tag based system for automatic purification and detection of soluble protein levels, alleviating problems associated with current protein preparation methods.

Detection methods for specifically monitoring recombinant protein levels, facilitating screening of expression and purification conditions, currently all have their limitations. Conventional fusion tag systems do not provide instant results and cannot be carried out online [3–5]. Mass spectrometry, chemiluminescence, nephelometry, and biosensors require complex equipment that cannot be integrated easily for chromatography and do not inherently allow a generic approach [6]. Only innately colored fusion tags seem to be universally applicable, allowing measurements using standard photometric equipment. Such a colored protein domain must, however,

* Corresponding author.

E-mail address: christian.ostermeier@pharma.novartis.com (C. Ostermeier).

consolidate robustness, high yield, solubility, and independence of host strains and cofactors, so as not to compromise potential fusion constructs or limit expression conditions. It further must not form aggregates, precipitate easily or otherwise encumber handling during protein purification. This has prompted us to compare a variety of intrinsically colored proteins that potentially might be used to spectroscopically monitor protein levels during purification. Parallel expression and affinity chromatography purification of Rubredoxin, Flavodoxin, and GFP established spectral properties and expression levels in a typical fusion context. We consequently focused on Rubredoxin as the ideal colored tag and evaluated its behavior and merits during expression level screening and different protein purification procedures.

Materials and methods

— Scott Lesley
works at Scripps
& collaborates
Novartis

A plasmid containing the Rubredoxin (Rub) gene from *Thermotoga maritima* was provided by S. Lesley, GNF, La Jolla, CA, USA. SuperglowGFP was obtained from Qbiogene. Flavodoxin (Flv) was cloned from genomic *Helicobacter pylori* DNA, provided by R. Paul, Biozentrum, Basle, Switzerland (Swissprot Q25776, with S44G, T78N, P117S, and S153A). CD11c I-domain was available in-house (Swissprot P20702, with Met-Ala preceding residues 141–348).

Preparation of plasmids was generally performed using classic enzymatic restriction based cloning or sticky-end PCR [7], the latter making it possible for inserting any target gene into any of the Rub-tag vectors created, due to a consistent multiple cloning site (MCS; *NcoI/BamHI* for the target gene, Fig. 2A). For insertion of Rub and a PreScission protease cleavage site (PCS) flanked MCS into new expression vectors, we resorted to a recently described PCR based method [8]. Such a step is required only once for inserting our expression system into an entirely new vector backbone, while the N-terminal tag, PCS or even color tag can be exchanged using classic restriction and ligation. Seamless target gene insertion via two consistent restriction sites (*NcoI/BamHI*) into constructs with varying N- and C-termini, including the original N-terminus, requires only one single donor plasmid, supporting extensively automated parallel screening. Further, constructs produced in parallel by excision from a verified donor plasmid require no resequencing as insertion is by ligation, not PCR.

Cell-free expression was performed according to manufacturer's guidelines (Roche), with the exception of adding iron (II) citrate to a final concentration of 6.8×10^{-4} M to the reaction mixture for enhanced iron incorporation into Rub (using a 0.2 μ m filtered 0.15 M FeSO_4 , 0.75 M Na_3 citrate solution).

Initial capturing steps using Superflow Ni-NTA resin were performed according to manufacturer's guidelines

(Qiagen). A Beckman-Coulter DU-800 photospectrometer was used for absorption measurements, using the corresponding protein-free buffer for baseline correction; Rub shows two absorption maxima at 380 and 490 nm, with averaged $\epsilon_{380}(\text{Rub-fusion}) = 4.67 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{490}(\text{Rub-fusion}) = 3.75 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ when Rub is fused to CD11c in different contexts (Ni-NTA and HPLC purified). Concentration determinations via HPLC were performed by BSA-calibrated absorption monitoring at 220 nm on a Poros R1/10 column (Applied Biosystems).

Initial color tag comparisons were performed using constructs containing an N terminal His tag, followed by the CD11c gene, with C-terminal Rub, Flv, or GFP (276, 386, and 461 aa, respectively, for the entire fusion protein). The final Rub expression and detection system was constructed differently: A consistent MCS allowed free exchange of N-terminal transcription-, translation-, or solubility enhancing tags as deemed appropriate, while the affinity chromatography handle and the detection unit were both C-terminal (Fig. 2A).

For anion exchange chromatography, we generally used Resource Q or Source 30 Q resin from Amersham Biosciences. Proteins were eluted with a linear gradient of 0–50% 1 M NaCl in 50 mM Tris-HCl, pH 8.0.

Results and discussion

Based on reported biochemical properties, we initially came to the following conclusions: Copper proteins often contain hydrophobic patches and are produced as apoprotein, requiring incubation with Cu^{2+} to produce holoprotein [9]. Ferredoxins likewise may require reconstitution and form dimers [10].

Biliproteins and cytochromes require heme synthesis and processing capabilities, and thus, are dependent on additional genetic elements [11,12].

Due to its widespread use, we retained GFP for a comparison with proteins that were better suited to the task in spite of GFP inclusion body formation at temperatures above 30°C and low yields [13]. Bacterial cells expressing the red fluorescent, tetrameric protein DsRed show growth inhibition and protein aggregation [14]. All GFP variants, with their fundamental β -barrel structure and corresponding molecular weight of ~27 kDa, place a disproportionate burden on the prokaryotic expression system, with the tag typically as large as the target gene.

Finally, Flavodoxin (Flv), and especially Rubredoxin (Rub) remained as candidates, with reported high yields and satisfactory sensitivity [2,15]. Flv (17.6 kDa) is smaller than GFP, but Rub (6.1 kDa) alone deserves the designation "tag". The Rub chromophore is the most simple of all FeS clusters, Fe^{3+} tetrahedrally coordinated by 4 cysteines, embedded in a highly compact and stable globular domain [16].

For an experimental comparison, superglowGFP (Qbiogene), Flv from *H. pylori*, and Rubredoxin (Rub) from *T. maritima* were fused to the C-terminus of the human CD11c I-domain (the insertion domain from leukocyte adhesion glycoprotein P190.95), expressed in *E. coli*, and purified via N-terminal His-Tag. Table 1 summarizes the obtained results: It seems that there is a trade-off between detection sensitivity and soluble expression yield. We found that, when fused to GFP, CD11c was produced at levels an order of magnitude lower than when using Rubredoxin as the fusion partner. Optical absorption of the Rub construct was 17 times lower. However, structural genomics requires mg protein amounts where high sensitivity is non-essential but high soluble expression levels in the mg range are crucial. Additionally, the sensitivity of Rubredoxin is sufficient for tracking less than 100 µg of total protein during chromatography ($\epsilon_{380}(\text{Rub-fusion}) = 4.67 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$), and thus, is ideal for structural genomics applications.

While GFP variants other than superglowGFP may express differently, the 11-stranded β -barrel intrinsically is quite large (~27 kDa) [17], likely limiting soluble expression levels, as has been demonstrated recently for other proteins [18]. Further, both Flv and GFP limited expression conditions to temperatures around 30 °C as opposed to 37 °C for Rub, otherwise resulting in strong inclusion body formation. As described below, Rub did not handicap protein preparation in any way.

Thus, and especially in view of Rubredoxin resulting in the highest target protein yields of the three candidates,

we decided to exclusively implement Rubredoxin in the final expression system (Fig. 2A for a schematic of the expression plasmid backbones). We additionally characterized aspects that were relevant to protein preparation and purification using Rub/His in a pET24a vector: Expression levels of ~50 mg native protein per liter (7.2 µmol/l) of standard shake flask culture and a final OD₆₀₀ of 2.5 at 37 °C in LB media showed Rub to be non-toxic and highly expressible in a soluble form. Robustness in conditions typically encountered during protein purification also was a central issue. pH extremes were assessed in view of ion exchange chromatography, reducing agents for potentially disrupting color by reducing Fe³⁺ to Fe²⁺ and a chelating reagent to verify stability of the chromogenic ion. Even after prolonged incubation, all samples predominantly retained their color. This, together with Rub's high thermostability, suggests that most target proteins will actually be far more fragile than the Rubredoxin "detection unit" (Table 2).

Of further interest was the behavior of Rub fusion proteins during chromatography. We never observed dimerization, aggregation, or precipitation with any of the fusion proteins constructed according to Fig. 2A (T7tag/CD11c/Rub/His, GST/CD11c/Rub/His, CD11c/Rub/His, Rub/CD11c/His, and VLA2-I-domain/CD11c/Rub/His). Rub is produced as two species in *E. coli*: a colored fraction containing Fe³⁺ and a non-colored fraction with Zn²⁺ [2]. The two species only were separable during high-resolution ion exchange chromatography. When resolved, the two fractions were coupled

Table 1
Rub, Flv, and GFP as fusion partners for CD11c I-domain expression

Characteristic	His/CD11c/Rub	His/CD11c/Flv	His/CD11c/GFP
Number of residues/MW in kDa	276/31.0	386/42.4	461/51.9
CD11c yield from 250 ml LB (mg)	6.72 (37 °C)	6.24 (30 °C)	0.826 (30 °C)
Extinction coefficient (M ⁻¹ cm ⁻¹)	$\epsilon_{380} = 5.00 \times 10^3$ $\epsilon_{490} = 4.09 \times 10^3$	$\epsilon_{380} = 4.10 \times 10^3$	$\epsilon_{475} = 6.81 \times 10^4$
Inclusion bodies at 37 °C	—	+	+++
Additional reagents/cofactors required for chromophore formation?	(Fe ³⁺ /H ⁺ , present in LB medium)	No	No

Note. Expression under identical conditions (except for temperature, as only Rub remains largely soluble at 37 °C). The materials and methods section describes yield determination of Ni-NTA purified proteins by analytical HPLC, as well as the measurement of extinction coefficients by spectroscopy.

Table 2
Stability of Ni-NTA purified Rubredoxin from *Thermotoga maritima*

Buffer	Temperature (°C)	Incubation time (h)	% Functional Rub
pH 7.0	70	4	>50
pH 9.5	20	12	62
pH 3.5	20	12	54
10 mM DTT, pH 7.0	20	24	81
10 mM β -ME, pH 7.0	20	24	68
10 mM EDTA, pH 8.0	4	40	99

Note. In all cases, buffer additionally contained 300 mM NaCl and 200 mM imidazole (Ni-NTA elution buffer for immobilized metal affinity chromatography). Rub concentrations were adjusted to 1 mg/ml. Functional amounts of Rub were determined according to residual absorption at 380 nm.

and gave a characteristic pattern with a consistent ratio [2] that could not be influenced by supplementing medium with chelated iron (data not shown). When expressed *in vitro* (Roche RTS HY 500), where the Fe^{3+} concentration during translation could directly be controlled, only the red Fe^{3+} -species was observed.

In an attempt to establish specificity of the Rub signal, we selectively isolated a T7tag/CD11c/Rub/His fusion protein from crude cell lysate. Purification was monitored using Rub absorption maxima at 380 and 490 nm, i.e., the tag's red color. Two subsequent IEX steps resulted in a higher purity than when Ni-NTA affinity chromatography alone is used (Fig. 3A, final two lanes). Combining the signal specificity with affinity chromatography, an initial capturing step including buffer exchange may easily be completely automated by defining an absorption threshold for peak collection and redirection, as we were able to verify using the Äkta 3D Explorer system from Amersham Biosciences (Fig. 3A, first two lanes, cf. also Fig. 3B). This will allow fully automated purification of dozens of proteins per day and chromatography station, including discrimination of colored target over contaminating unselectively bound proteins.

Tracking the unique color of Rubredoxin fusion proteins thus will allow automation of both generic (affinity tag) and non-generic, conventional (ion exchange, size exclusion, etc.) chromatography steps. Selection and redirection of the colored peaks also allow automation of rapid multi-step chromatography procedures without intermittent time-consuming analysis steps.

We also evaluated whether the spectral properties of Rub expressed in *E. coli* would enable reliable absolute quantitation of varying fusion constructs. By comparing analytical HPLC data [19] with Rub based protein estimation, this was shown to be the case, regardless of whether Rub was N- or C-terminal in the fusion protein (Fig. 3C). Additionally, the ratio of the two absorption

maxima of a Rub fusion protein (Fig. 1A) may function as a preliminary quality test of a given sample prior to more extensive functional analyses.

A recent study of 32 human proteins conclusively showed the importance of N-terminal variation of fusion tags for protein expression and solubility [1]. Therefore, we designed constructs with C-terminal Rub for total flexibility of N-terminal sequence. Additionally, we found that Rub fused to the N-terminus of CD11c resulted in a 200% higher yield than that obtained with homologous constructs containing a T7tag or the CD11c original N-terminus. In view of the highly hydrophilic water accessible surface area of Rub (Fig. 1B top) and these initial data, we will be including both N- and C-terminal Rub in all our future expression constructs.

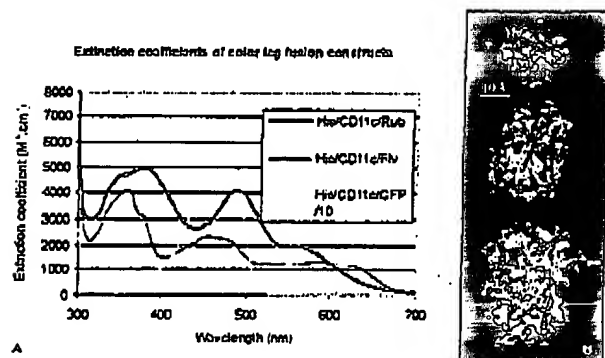


Fig. 1. Comparison of Rub, Flv, and GFP as color tags: (A) Vis-spectra overlay of fusion proteins containing Rub, Flv, and GFP (GFP scaled down by factor of 10 for visibility). (B) Comparison of Rub, Flv, and GFP size (all drawn to scale, bar corresponds to 10 Å). Hydrophobic (dark blue) and hydrophilic (red) residues mapped to water accessible surface area.

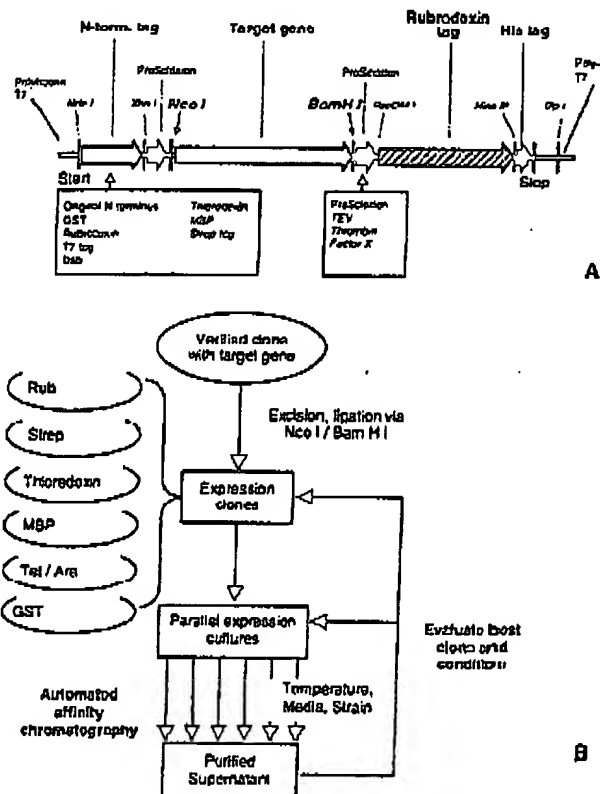


Fig. 2. Ruby Tag system and workflow. (A) The common backbone, where the N-terminal tag can be exchanged at leisure and any target gene easily is inserted via *NcoI/BamHI* restriction sites. The Rub detection unit and His affinity chromatography handle are N-terminal. Suggested but not implemented features are in italics. (B) Target genes are inserted into a "molecular biology vector" of choice by any method. From a large pool of a sequence-verified clone, the target is excised and simultaneously inserted into an array of relevant expression vectors. Parallel expression is followed by automatic affinity chromatography (Fig. 3A) and simple spectroscopic readout of relative protein levels, efficiently screening for optimal expression conditions and clones. Finally, the Rub signal facilitates dedicated purification (Figs. 3A and B). Processes in bold font are made easier using the Rub tag.

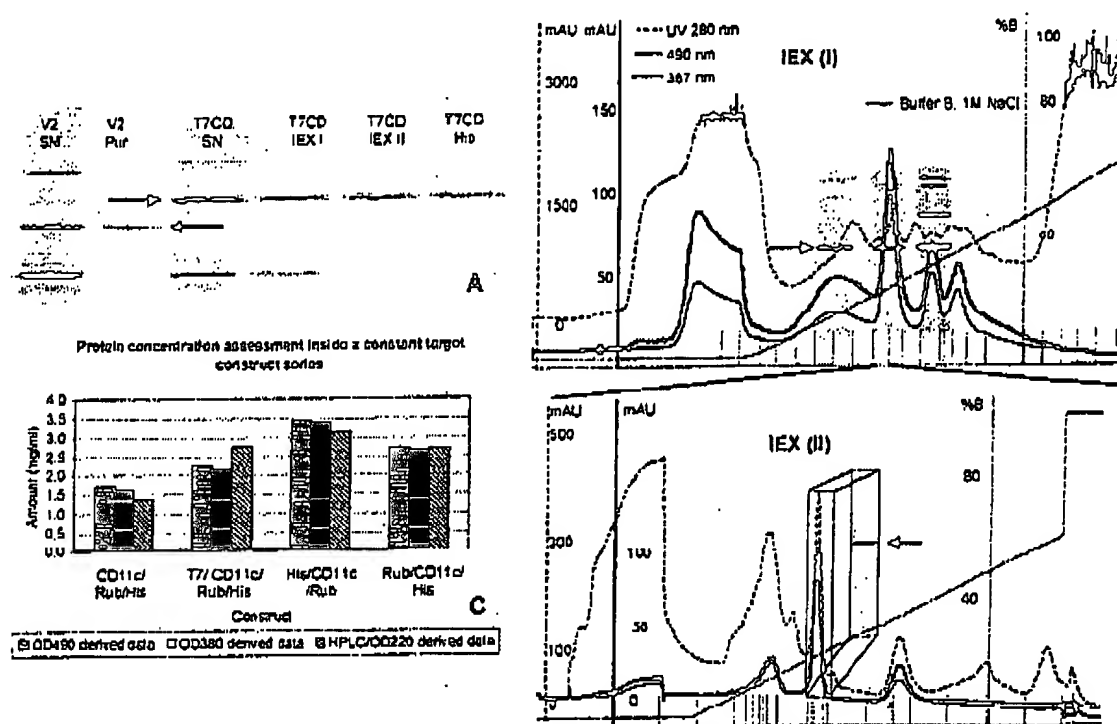


Fig. 3. The use of Rub for purification. (A) VLA2/Rub/His purified automatically on a multi-dimensional chromatography system, with peak collection and redirection (Ni-NIA, desalting) based on Rub absorption at 380 nm. T7/CD11c/Rub/His purified without affinity chromatography, based on the same signal. V2SN: VLA2/Rub/His lysed cell supernatant (SN). V2Pur: V2SN automatically purified as described. T7CDSN: T7/CD11c/Rub/His lysed cell supernatant. T7CD IEX I and II: T7CDSN, after first and second round of anion exchange purification, respectively. T7CDHis: T7/CD11c/His lysate purified by affinity chromatography, comparing unfavorably with the previous lanes, purified guided by Rub signal alone (albeit in two steps, see B). (B) Chromatograms of the two anion exchange steps that were used to successively purify crude cell lysate containing T7/CD11c/Rub/His, resulting in the samples shown in (A). Note the Rub signals and their common absorption axis depicted in solid lines. SDS-PAGE insets correspond to the overlying peaks in the top chromatogram and the indicated major peak in the bottom chromatogram. They allow qualitative comparison of purity, while applied sample amounts differ. (C) Comparison of analytical HPLC- and Rub-determined protein amounts from samples with different tags and N- and C-terminal Rub. Arrows denote target fusion protein.

It may be interesting to note that Rub retained its color, even when the target protein was precipitated by low ion concentrations or heat denaturation. Further, $^1\text{H-NMR}$ monitoring of CD11c at different temperatures showed Rub to act as an independent domain: CD11c/Rub/His contained spectral features that were characteristic of untagged CD11c. After heat treatment of the fusion protein CD11c/Rub/His at 43 °C, CD11c signals disappeared, while Rub spectral features remained (data not shown). This suggests an additional use of Rub as a solubility enhancing tag during NMR experiments, where prior tag cleavage might potentially pose problems.

Conclusion

In summary, we propose Rubredoxin as a standard fusion tag for online detection of soluble protein levels during generic and dedicated protein purification. We have demonstrated its robustness, practical use in protein quantitation, and tracking as well as its high po-

tential for automated purification. Large-scale structural genomics and proteomics projects require generic approaches to protein preparation, which are often in conflict with the quality or yield of final purified protein. Here, we have demonstrated the "RubyTag" to be a pragmatic alternative, which is amenable to carefree automatic purification and handling due to its high expression levels and robust biochemical properties. Finally, thanks to the real-time detection and tracking possibility of Rub-tagged fusion proteins, parallel screening of a multitude of constructs and expression conditions, as well as rapid optimization of the entire protein preparation process, can be streamlined using the RubyTag (Fig. 2B).

References

- [1] M. Hammarström, N. Hellgren, S. van Den Berg, I.-I. Berglund, T. Hard, Rapid screening for improved solubility of small human proteins produced as fusion proteins in *Escherichia coli*, Protein Sci. 11 (2002) 313–321.

- [2] I. Mathieu, J. Meyer, J.M. Moulis, Cloning, sequencing and expression in *Escherichia coli* of the rubredoxin gene from *Clostridium pasteurianum*, *Biochem. J.* 285 (Pt 1) (1992) 255–262.
- [3] R.T. Raines, M. McCormick, T.R. Van Oosbree, R.C. Mierendorf, The S-Tag fusion system for protein purification, *Methods Enzymol.* 326 (2000) 362–376.
- [4] A. Skerra, T.G. Schmidt, Use of the Strep-Tag and streptavidin for detection and purification of recombinant proteins, *Methods Enzymol.* 326 (2000) 271–304.
- [5] D.B. Smith, K.S. Johnson, Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase, *Gene* 67 (1988) 31–40.
- [6] K.N. Baker, M.H. Rendall, A. Patel, P. Boyd, M. Hoare, R.B. Freedman, D.C. James, Rapid monitoring of recombinant protein products: a comparison of current technologies, *Trends Biotechnol.* 20 (2002) 149–156.
- [7] G. Zeng, Sticky-end PCR: new method for subcloning, *Biotechniques* 25 (1998) 206–208.
- [8] M. Geiser, R. Cebe, D. Drewello, R. Schmitz, Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase, *Biotechniques* 31 (2001) 88–90, see also p. 92.
- [9] A. Bengrine, N. Guilian, C. Appia-Ayme, E. Jedlicki, D.S. Holmes, M. Chippaux, V. Bonnefoy, Sequence and expression of the rusticyanin structural gene from *Thiobacillus ferrooxidans* ATCC33020 strain, *Biochim. Biophys. Acta* 1443 (1998) 99–112.
- [10] P. Rodrigues, F. Graca, A.L. Macedo, I. Moura, J.J. Moura, Characterization of recombinant *Desulfohalobium gigas* ferredoxin, *Biochem. Biophys. Res. Commun.* 289 (2001) 630–633.
- [11] N.J. Price, L. Brennan, T.Q. Faria, E. Vijgenboom, G.W. Canters, D.L. Turner, H. Santos, High yield of *Methylophilus methylotrophus* cytochrome c by coexpression with cytochrome c maturation gene cluster from *Escherichia coli*, *Protein Expr. Purif.* 20 (2000) 444–450.
- [12] A.J. Toolcy, Y.A. Cai, A.N. Glazer, Biosynthesis of a fluorescent cyanobacterial C-phycoerythrin holo-alpha subunit in a heterologous host, *Proc. Natl. Acad. Sci. USA* 98 (2001) 10560–10565.
- [13] C.R. Narahari, L. Randers-Eichhorn, J.C. Strong, N. Ramasubramanian, G. Rao, D.D. Frey, Purification of recombinant green fluorescent protein using chromatofocusing with a pH gradient composed of multiple stepwise fronts, *Biotechnol. Prog.* 17 (2001) 150–160.
- [14] S. Jakobs, V. Subramaniam, A. Schonle, T.M. Jovin, S.W. Hell, EGFP and DsRed expressing cultures of *Escherichia coli* imaged by confocal, two-photon and fluorescence lifetime microscopy, *FEBS Lett.* 479 (2000) 131–135.
- [15] R. Paul, F.U. Busch, K.P. Schaefer, Overexpression and purification of *Helicobacter pylori* flavodoxin and induction of a specific antiserum in rabbits, *Protein Expr. Purif.* 22 (2001) 399–403.
- [16] F. Bonomi, D. Fozzas, S. Iametti, D.M. Kurtz, S. Mazzini, Thermal stability of *Clostridium pasteurianum* rubredoxin: deconvoluting the contributions of the metal site and the protein, *Protein Sci.* 9 (2000) 2413–2426.
- [17] M. Zimmer, Green fluorescent protein (GFP): applications, structure, and related photophysical behavior, *Chem. Rev.* 102 (2002) 759–781.
- [18] P. Braun, Y. Hu, B. Shen, A. Halleck, M. Koundinya, E. Harlow, J. LaBac, Proteome-scale purification of human proteins from bacteria, *Proc. Natl. Acad. Sci. USA* 99 (2002) 2654–2659.
- [19] T.G. Heath, A.B. Giordani, Reversed-phase capillary high-performance liquid chromatography with on-line UV, fluorescence and electrospray ionization mass spectrometric detection in the analysis of peptides and proteins, *J. Chromatogr. A* 638 (1993) 9–19.